World Cancer Research Journal

WCRJ 2026; 13: e3032
DOI: 10.32113/wcrj_20265_3032

CHANGES IN OSTEOSARCOMA MICROENVIRONMENT CONTRIBUTE TO DOX RESISTANCE

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ABSTRACT – The osteosarcoma tumor microenvironment has both cellular and non-cellular components. Among the cellular components, both immune and non-immune cells can be derived. The immune cells include infiltrating T lymphocytes, B cells, and dendritic cells, while the non-immune cells include macrophages and cancer-associated fibroblasts. The extracellular matrix, blood vessels, and all cytokines and chemokines are included in the non-cellular portion. Hypoxia-inducible factor-1α (HIF-1α) is up-regulated during the early stages of tumor formation due to an induced hypoxic drive. Tumor suppression, progression, or relapse may result from the interplay of various elements in the tumor microenvironment.

Tumor suppression occurs when immune cells cooperate to eliminate cancer cells, whereas tumor formation occurs when an immunosuppressive microenvironment is created. Tumor growth or recurrence might result from some components of the tumor microenvironment releasing chemicals that hinder other immune cells from defending the tumor. T regulatory cells release the inhibitory cytokines interleukin (IL)-10 and IL-35, produce bioactive transforming growth factor beta (TGF-β), and induce death in effector T cells. Macrophages and dendritic cells produce HIF-1α, which directly reduces tumor cells' vulnerability to doxorubicin (DOX)-induced apoptosis. Doxorubicin, narasin, ifosfamide, cisplatin, methotrexate, gemcitabine, etoposide, carboplatin, and cyclophosphamide have all been used to treat osteosarcoma. Modest doses of doxorubicin are recommended because of the cardiotoxic effects alone, but these leave some cancer cells alive. This review explicitly describes how the changes in the tumor microenvironment contribute specifically to doxorubicin resistance and how doxorubicin-resistant cells affect the components in the tumor microenvironment.

KEYWORDS: Sarcoma, Doxorubicin, Chemotherapy, Osteosarcoma, Tumor microenvironment, Drug resistance, Immunotherapy, Immune cells, Macrophages, Cancer-associated fibroblasts.

ABBREVIATIONS: ACT: Adoptive cell transfer, APCs: Antigen-presenting cells, CAFs: Cancer-associated fibroblasts, CTLA-4: Cytotoxic T-lymphocyte–associated protein 4, CTLs: Cytotoxic T lymphocytes, DOX: Doxorubicin, IFNs: Interferons, mregDCs: Mature immunoregulatory dendritic cells, PD-1: Programmed cell death protein 1, PD-L1: Programmed death-ligand 1, TAMs: Tumor-associated macrophages, TGF-β: Transforming growth factor beta, TNF: Tumor necrosis factor, TRAIL: TNF-related apoptosis-inducing ligand, Tregs: Regulatory T cells.

INTRODUCTION

Bone cell formation requires growth hormones, cytokines, enzymes, collagen and non-collagenous proteins to encourage differentiation[1]. Under certain conditions, such as the accumulation of mutations or gene fusions, normal bone cells can develop into cancerous cells, causing osteosarcoma[2]. The condition initially peaks between the ages of 10 and 14, during pubertal growth spurts, although late-onset disease is also reported. Osteosarcoma creates high vascular density to maintain its malignancy, which makes it more aggressive[3]. According to reports[4-5], worse prognoses are linked to obesity, fractures, and patient aging. Over the years, a variety of therapeutic agents have been utilized to treat osteosarcoma, including doxorubicin (DOX), narasin, ifosfamide, cisplatin, methotrexate, gemcitabine, etoposide, carboplatin, and cyclophosphamide. However, doxorubicin has proven to be more successful and widely used[6-8]. Although doxorubicin has shown promise, its cardiotoxicity impacts the drug application as a chemotherapeutic agent[9]. Overall, it breaks DNA strands, prevents topoisomerase from functioning, and initiates the production of reactive oxygen species, all of which worsen cell damage[10]. In addition to directly targeting cancer cells, doxorubicin promotes the maturation of antigen-presenting cells (APCs), such as tumor macrophages and dendritic cells, and the infiltration of immune cells, such as T cells (CD8+) and natural killer cells[10]. These cells, together with other components like fibroblasts, cytokines, and chemokines, form the tumor microenvironment in osteosarcoma[11].

The tumor microenvironment has both cellular and non-cellular components[12]. Blood arteries and extracellular matrix comprise the non-cellular components, whilst immune and non-immune cells make up the cellular components[13]. The presence of each component serves as a predictive tool to identify targets for suppressing osteosarcoma and to determine prognosis. Each cell plays a distinct role in either promoting or suppressing tumor growth[14-16]. In a malignant condition, the immune component of the tumor microenvironment, such as T cells, regulatory T cells, NK cells, B cells, and dendritic cells[17,18], whether activated or inactivated, exhibits a variety of phenotypic traits, releases cytokines and chemokines, and causes infiltration of the tumor site[19].

IMMUNE CELLS OF THE OSTEOSARCOMA MICROENVIRONMENT

T cells

T cells are the major cell type infiltrating the tumor microenvironment to exert maximal tumor suppression[20]. The populations of T cells that infiltrate the tumor site are the cytotoxic CD8+ and helper CD4+ cells[21]. The helper CD4+ cells can exert direct effector functions, but their presence mainly stimulates CD8+ cells to fight the tumor[22]. The cytotoxic CD8+ cells then release their granules, kill an infected cell, and then move to a new target and kill again, often referred to as serial killing[23]. The effectiveness of cytotoxic CD8+ cells is often hindered by immunosuppressive factors in the tumor microenvironment (TME), leading to T cell exhaustion. In this case, there are inactive CD8+ cells that have lost function[24]. Aside from this cell exhaustion, checkpoint proteins such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or the programmed cell death 1 (PD-1) and programmed death-ligand 1 (PD-L1) pathways have been observed to hinder successful recognition of tumor-associated antigens and consequent eradication of cancer by active cytotoxic T lymphocytes (CTLs)[25-28]. Strategies such as adoptive cell transfer (ACT), genetic modification of T cells, and immune checkpoint inhibitors are being developed to overcome these challenges and harness T cells for improved osteosarcoma treatment[29]. Phenotypic studies[28,30] and the quantification of tumor-infiltrating lymphocyte subsets have demonstrated the immune system's capacity, implying their involvement in modulating cancer progression and predicting responses to immunotherapies. However, cumulative evidence indicates a predominant infiltration of exhausted CD8+ T cells in primary osteosarcoma tissues. The functional activation of tumor-infiltrating CD8+ T cells *in vivo* has been shown to achieve maximum tumor suppression, as claimed by a study[31], but these cells are mostly found in lower numbers at the tumor site. Activated CD4+ and CD8+ T cells can withstand the cytotoxic effects of doxorubicin. Doxorubicin has not been shown to induce cytotoxicity in T cells, except for enhancing the functionality of CD8+ T cells. Regulatory T cells, instead, secrete immunosuppressive cytokines and upregulate immune checkpoints, shielding tumor cells from elimination by doxorubicin.

Regulatory T cells

Regulatory T cells (Tregs) play a suppressive role in the immune system component of the tumor microenvironment, maintaining immune homeostasis and preventing excessive immune responses[32]. Tregs can hinder anti-tumor immune responses in the cancer environment by suppressing cytotoxic T cells and other immune cells and promoting immune evasion by cancer cells[33,34]. Immune evasion is a strategy cancer cells use to evade immune surveillance and evade attack[35]. They suppress T cell function by producing the inhibitory cytokines IL-10 and IL-35, delivering bioactive TGF-β, and inducing the apoptosis of effector T cells. This suppression is achieved by IL-2 depletion *via* high-affinity IL-2Ra (CD25)[36]. Indirect suppressive mechanisms involve the elimination of antigen-MHCII and CD80-CD86 through T-cell receptor and CTLA-4-mediated transendocytosis and trogocytosis events[37,38]. Tregs are also known to suppress NK cell activity through the secreted factors, TGF-β and IL-10, and by upregulating PD-L1, which can dampen NK cell function and contribute to immune evasion and tumor progression. Doxorubicin treatment is less effective when an absolute immunosuppressive and pro-tumorigenic milieu is created by high Treg levels in the tumor microenvironment.

NK CELLS

The NK cells can eliminate cancer cells through complex mechanisms like releasing cytotoxic granules containing perforin, granzymes, and granulysin cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, to activate antitumor immunity and death ligands, such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL)[39,40]. They are a class of innate lymphoid cells recognized as non-specific cytotoxic immune cells[41]. These cells control tumor growth and metastasis without requiring prior activation or sensitization[39].

In osteosarcoma, NK cells are a common tumor-infiltrating lymphoctes subset that can be classified into two subclusters based on NK cell marker expression (NKG7 and GNLY)[42]. One subcluster, expressing the T cell markers CD3D and CD8A, is classified as NK T cells. These cells show activation and a strong expression of GZMB, GZMA, and IFN-γ, indicating tumor cytotoxicity in osteosarcoma[43]. The other subcluster, classified as NK cells, has only a small fraction expressing GZMB, IFN-γ, and PRF1, suggesting a non-activated state in osteosarcoma lesions[44]. Doxorubicin and NK cells interact in a complex way. At large dosages, doxorubicin can either cause NK cell dysfunction or make tumor cells more susceptible to NK-mediated destruction. Dendritic cells aid NK cell development, and doxorubicin can impede dendritic cell maturation. Both NK-cells and dendritic cells have a bidirectional and cooperative relationship, with each cell type regulating the other's maturation and function to coordinate immune responses against infections and tumors[45]. Dendritic cells (DCs) can activate resting NK cells, prompting them to kill target cells, while activated NK cells can eliminate immature DCs and promote their maturation[46].

B cells

The B cells have a dual role in osteosarcoma. They are capable of both promoting and suppressing tumor progression[47]. Their functions include producing antibodies and cytokines, presenting antigens, and influencing other immune cells. The presence of specific B cell populations is associated with the tumor microenvironment and may impact patient prognosis and response to immunotherapies[48]. B cells primarily present tumor antigens to T cells, which primes the anti-tumor response, and secrete anti-tumor antibodies and cytokines that enhance the activity of other immune cells, such as NK cells and cytotoxic T cells[49]. Regulatory B cells, on the other hand, secrete immunosuppressive cytokines like IL-10 and TGF-β, and express regulatory molecules such as FasL and CD1d[50]. B regulatory cells can also promote the accumulation of Tregs and directly inhibit CD4+ T cells and cytotoxic T lymphocytes, ultimately weakening the anti-tumor immune response needed to fight osteosarcoma. Other cytokines are known to enhance anti-tumor immunity and are part of the non-immune components of the tumor microenvironment.

DENDRITIC CELLS

Matured dendritic cells account for less than 5% of the total tumor-infiltrating myeloid cells in the tumor microenvironment of osteosarcoma. These are antigen-presenting cells that influence the development of adaptive immune responses, such as T helper 1 cells[51,52]. Dendritic cells capture tumor antigens, present them to T cells, and initiate an anti-tumor immune response. However, an analysis of a single-cell atlas of osteosarcoma and myeloid cells revealed that mature immunoregulatory dendritic cells (mregDCs) play a significant role in suppressing antitumor immunity in osteosarcoma[53,54]. The mregDCs, expressing CCR7, LAMP3, and CD83, interact with Tregs through CD274-PDCD1 and PVR-TIGIT signaling, as well as their physical juxtaposition. The role of mregDCs in recruiting Tregs, leading to an immunosuppressive microenvironment, has been observed in various cancer types[55,56]. mregDCs exert immunosuppressive functions by promoting the migration of Tregs into the TME[55]. These mregDCs interact with Tregs through CCR4 binding and the CXCL9/10-CXCR3 axis, among other mechanisms[57]. Dendritic cells (DCs) and B cells cooperate in immune responses as they both present antigens to T cells. There is a dynamic interplay where B cells can transfer antigens to dendritic cells for enhanced presentation and activated B cells can educate dendritic cells to promote specific T helper cell responses, leading to antibody production and regulation of immune activity. By evading the cytotoxic effects of doxorubicin, dendritic cells indirectly contribute to drug resistance. This suggests that the tumor microenvironment is continuously immunosuppressive, which leads to tumor growth.

NON-IMMUNE CELLULAR COMPONENTS OF THE OSTEOSARCOMA MICROENVIRONMENT

Tumor-Associated Macrophages

Macrophages can exhibit different phenotypes depending on their activation state, with M1 macrophages having anti-tumor properties and M2 macrophages having immunosuppressive properties[58]. First, monocytes play a crucial role in the tumor microenvironment, acting as a link between the innate and adaptive immune systems during cancer development[59]. They exhibit diverse functions in both pro-tumoral and anti-tumoral immunity, such as phagocytosis, lymphocyte recruitment, angiogenesis, and differentiation into tumor-associated macrophages (TAMs) and monocyte-derived dendritic cells. Two subtypes of monocytes, classical (CD14+CD16-) and non-classical (CD14-CD16+), show distinct functions in osteosarcoma[60]. In primary osteosarcoma tissues, classical (CD14+D16-) monocytes with an overexpression of VCAN and S100A8/9/12 exhibit pro-inflammatory functions, whereas the non-classical (CD14-D16+) monocytes with high levels of CDKN1C, LILRB2, TGAL, and CX3CR1 expression exhibit the anti-inflammatory effects[61]. The phenotypes of TAMs are linked to clinical outcomes in osteosarcoma. TAMs expressing CD14 or CD163 are associated with improved overall survival and metastasis-free survival in multiple osteosarcoma cohorts. TAMs in osteosarcoma consist of a heterogeneity of sub-populations, classified as anti-tumor M1-polarized macrophages and pro-tumor M2-polarized macrophages[62]. TAMs infiltrate massively into osteosarcoma tissues and specific sub-populations are involved in a wide range of tumor progression pathways[63]. Primary osteosarcoma tissues consist of highly infiltrating M2-polarized TAMs, indicating poor prognosis, as M2 macrophages promote tumor progression. M1-TAMs interact with Tregs and exhausted CD8+ T cells through ligand receptors like LGALS9, PDCD1LG, CD274, and SP1[55], and they are helpful in tumor suppression. Zhou et al[64] also identified a high proportion of M2-like TAMs (CD163, MRC1, MS4A4, and MAF) in primary osteosarcoma patients receiving chemotherapy. Through M2-type polarization and cytokine production (IL-6, IL-10, M-CSF), TAMs increase doxorubicin resistance[64].Within the tumor microenvironment, these provide an immunosuppressive and pro-survival milieu. Doxorubicin can also be taken up by macrophages, which reduces the amount of medication that reaches cancer cells. M2 macrophages influence the mesenchymal-mesenchymal transition of fibroblasts, enhancing their reactivity and promoting their activation into cancer-associated fibroblasts (CAFs).

CANCER-ASSOCIATED FIBROBLASTS

Cancer-associated fibroblasts, in return, recruit and promote the differentiation of M2-polarized macrophages (which are tumor-promoting). M2 macrophages, reciprocally, further activate CAFs and influence the tumor microenvironment to facilitate epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) remodeling[65]. Activated fibroblasts dynamically stimulate cancer cells through factors

like IL-6 and SDF-1, a process that can also be initiated by macrophage activity. The interplay between CAFs and M2 macrophages, in conjunction with tumor cells, enhances tumor cell motility, invasion, and metastatic spread[66]. Again, CAFs and M2 macrophages cooperate to activate endothelial cells and their precursors, leading to the formation of new blood vessels (angiogenesis) needed for tumor growth and metastasis[67]. The cancer-associated fibroblasts are then classified as exemplary forms of non-immune cells that stimulate the proliferation and invasion of osteosarcoma cells, even though they are stromal cells whose primary function is to produce extracellular matrix to maintain tissue structure[68]. From another perspective, CAF subpopulations appear dysfunctional in advanced osteosarcoma because they lack the common functional genes inherited from fibroblasts[69]. CAFs cause doxorubicin resistance by creating an excess of extracellular matrix elements, such as collagen, which gets so dense that it prevents doxorubicin from penetrating the tumor core.

All these components contribute to the complex environment of osteosarcoma and in the presence of doxorubicin treatment, changes are observed (Figure 1).

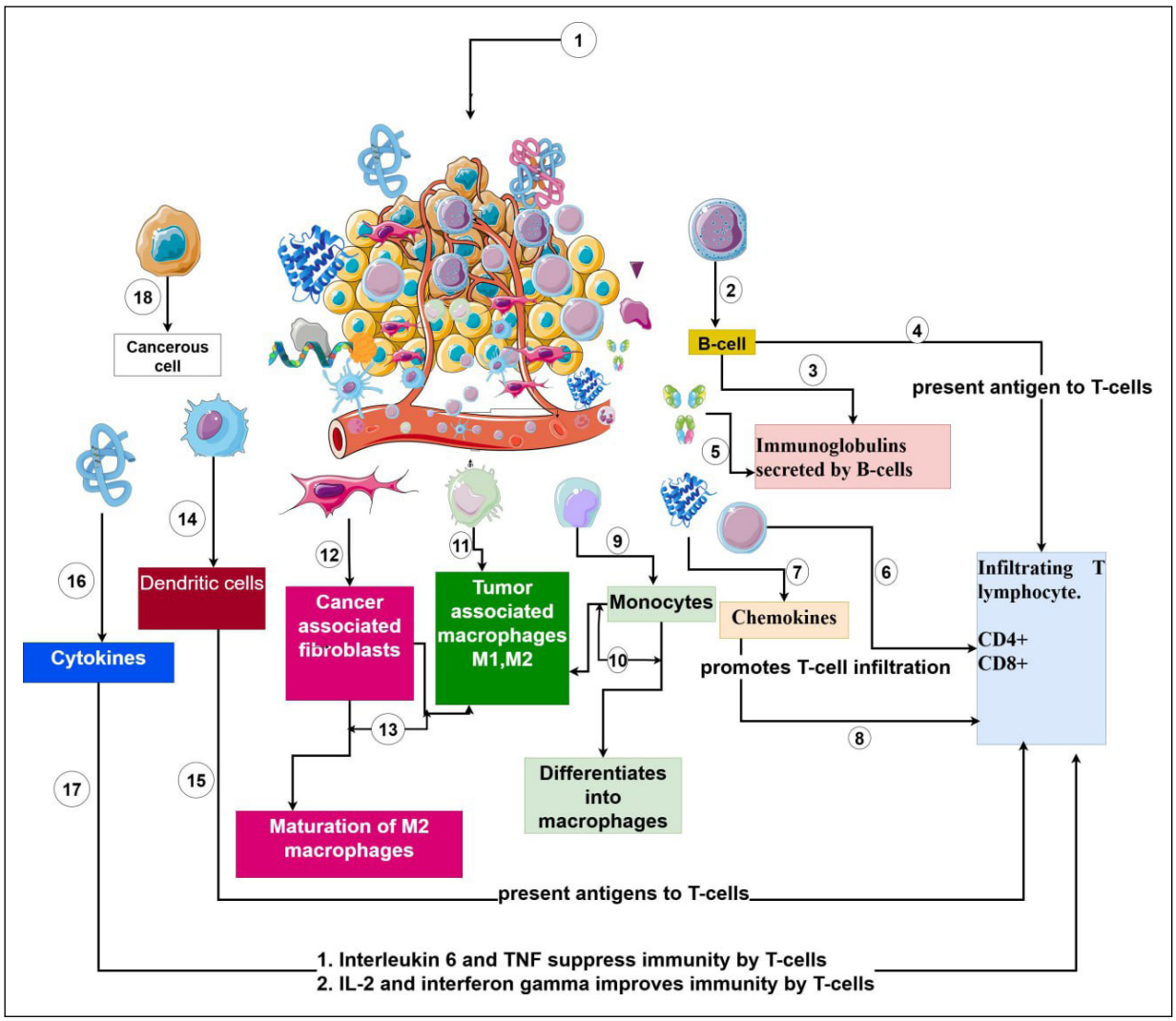

Figure 1. Representation of the osteosarcoma tumor microenvironment (created using BioRender). (1) Overview of the osteosarcoma tumor microenvironment, including angiogenesis and infiltration of immune and stromal cells. (2) B cell. (3) Immunoglobulins (IgM, IgG). (4) Antigen presentation to T cells. (5) Immunoglobulin secretion by B cells. (6) Infiltrating T lymphocytes (CD4+ and CD8+). (7) Chemokines. (8) Chemokine-mediated T-cell infiltration. (9) Monocytes. (10) Differentiation of monocytes into macrophages. (11) Tumor-associated macrophages (M1 and M2). (12) Cancer-associated fibroblasts. (13) Promotion of M2 macrophage maturation. (14) Dendritic cells. (15) Antigen presentation by dendritic cells to T cells. (16) Cytokines. (17) Cytokine-mediated modulation of T-cell immunity (suppression by IL-6 and TNF; activation by IL-2 and IFN-γ). (18) Cancer cell.

HOW DOES DOXORUBICIN AFFECT THE TUMORMICROENVIRONMENT?

Doxorubicin is an anthracycline. Multiple mechanisms have been proposed to explain the cytostatic and cytotoxic actions of anthracyclines[10,70]. These include free radical formation, lipid peroxidation, and direct membrane effects. DOX has the capability to intercalate into DNA, inhibit topoisomerase II, disrupt mitochondrial function, and potentiate free-radical generation and oxidative damage[71]. DOX causes the supercoiling of the DNA helix by intercalating into the DNA, untwisting the molecule, and resulting in positive supercoiling of the DNA helix. The formation of DOX–DNA adducts activates the DNA damage response (DDR) pathway[10]. During DNA replication and transcription, topoisomerases play a crucial role in maintaining the correct DNA structure. Supercoiled DNA emerging from DOX-DNA adducts unwinds and efficiently functions as a template upon the introduction of single- or double-strand breaks (SSBs or DSBs) by topoisomerase type I and II enzymes, respectively[10,72]. Topoisomerase II enzyme is possibly the main target of DOX; however, topoisomerase I inhibition may also play a role in DOX cytotoxicity. Low dosages (<1 μM) of DOX are thought to capture covalently bound topoisomerase II at DNA DSB sites and inhibit DNA religation. As a consequence of the induction of SSBs and DSBs in the DNA molecule, DOX further causes the upregulation of genes of the DDR pathway. The mechanism of action of DOX in initiating the production of reactive oxygen species (ROS) involves directly binding to cardiolipin on the inner mitochondrial membrane[73]. As a result, cardiolipin is incapable of acting as a cofactor for mitochondrial respiratory enzymes[74]. By doing this, it initiates the process of ROS production. An excessive generation of ROS can contribute to DNA damage through the action of radicals on DNA bases and the sugar-phosphate backbone. Unrepaired damage can lead to apoptosis, cell-cycle arrest, and senescence. Again, high amounts of ROS cause substantial damage to the mitochondrial structure, which ultimately results in cell apoptosis[75]. Cancer cells, in some cases, escape from DOX and one possible way is the removal of DOX from cancer cells using special transporters. Apart from DOX exerting direct cytotoxic effects on cancer cells, it also engages the immune system to kill cancer cells by triggering CD8+ T cell responses and the maturation of antigen-presenting cells (APCs), such as tumor macrophages and dendritic cells[76]. Moreover, pre-treatment with DOX in cancer patients is an effective strategy to boost anti-cancer immune responses by increasing antigen-specific CD4+ Th1 immune responses[10]. Another phenomenon is observed with DNA-damaging agents such as camptothecin and irinotecan, in which incubation with cancer cells leads to upregulation of PD-L1. PD-1 is constitutively expressed or activated in myeloid, lymphoid, normal epithelial cells, and cancer cells. Under normal conditions, it serves to suppress excessive immune cell activity that could otherwise cause tissue damage and autoimmune response. However, cancer cells utilize PD-L1 to avoid detection by the immune response, creating a limitation[77-80]. DOX induces all these changes in the tumor microenvironment, but at higher doses. DOX is cytotoxic to the heart; a search for ways to increase DOX efficacy in cancer cells while minimizing associated toxicities to non-cancerous tissues is at the forefront of scientific research. At a point, cancer cells become dormant and spike again through complex signaling pathways and environmental triggers, causing relapse[81]. When this happens, a high-grade osteosarcoma evolves, which demands more than just DOX as a treatment option. Major clinical problems opposing the cure of high-grade osteosarcoma are the presence of inherent or acquired drug resistance caused by the tumor microenvironment and the development of metastasis. In this regard, the need to test for more treatment options other than DOX arises (Figure 2).

DRUGS THAT TARGET THE TUMOR MICROENVIRONMENT

Clinical trials of various chemotherapeutic agents have been conducted to date. Narasin, a drug that has undergone clinical trials, is a polyether antibiotic widely used in veterinary medicine. It is shown that narasin is active against osteosarcoma cells at the same concentrations that are less toxic to normal cells[82]. This effect is achieved through growth inhibition and apoptosis induction, mediated by oxidative stress, damage, and mitochondrial dysfunction[83]. The combination of narasin and doxorubicin at non-toxic doses completely arrests osteosarcoma growth in mice, as further demonstrated in humans[84]. The concurrent administration of doxorubicin and narasin presents a viable alternative therapeutic approach for osteosarcoma. Also, functionalized nanocarriers and nanoparticles have been shown to be an effective strategy to protect drugs from rapid clearance, prolong their circulation time, and increase their concentration at tumor sites, thereby enhancing therapeutic efficacy and reducing side effects[85,86]. Biomimetic nanoparticles (DOX/siSUR-PLGA@MSCM NPs) have been synthesized by coloading DOX and survivin siRNA (siSUR) into poly (lactide-co-glycolide acid) (PLGA) *via* a double-emulsion solvent evap-

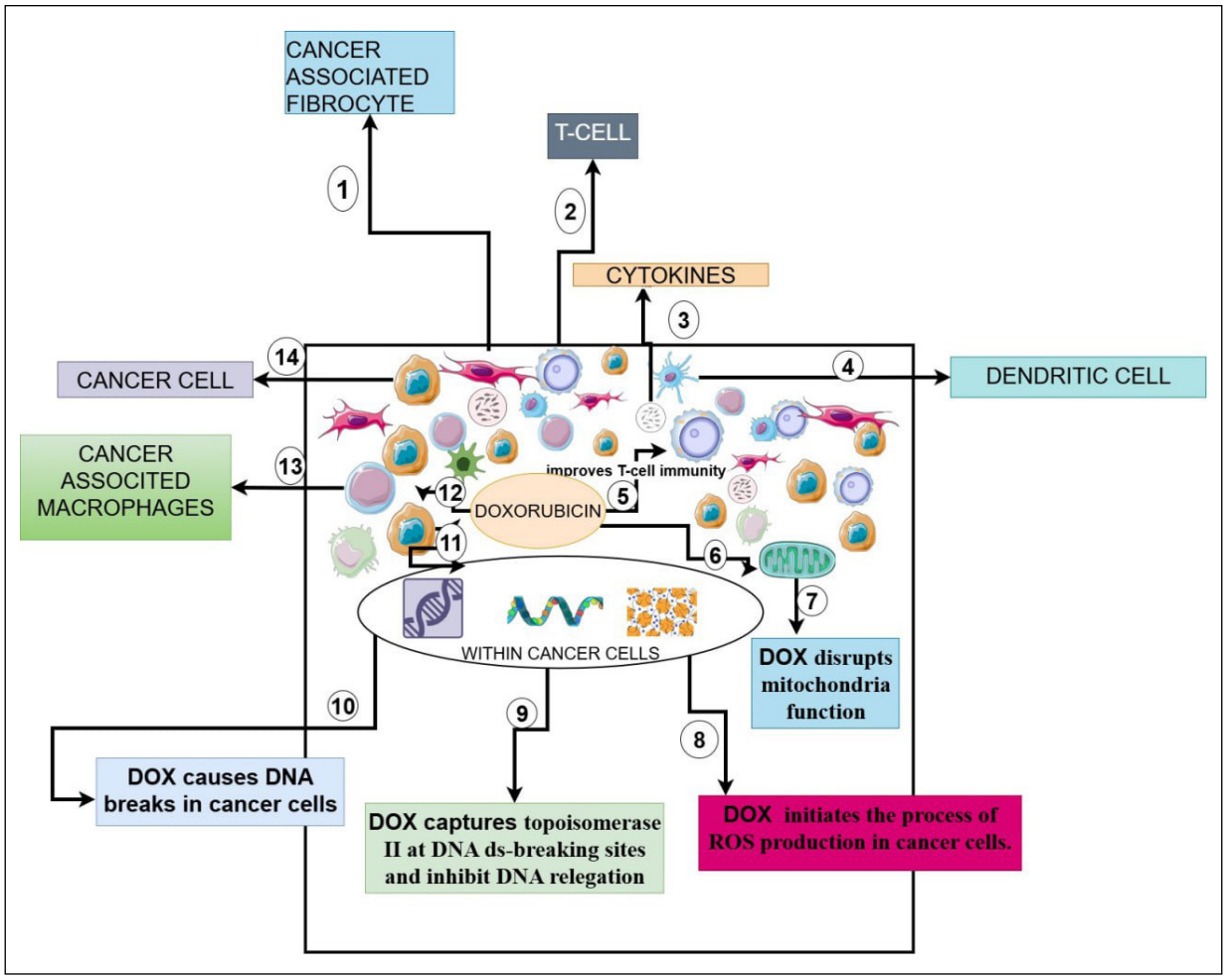

Figure 2. Effects of doxorubicin (DOX) on the tumor microenvironment. (1) Cancer-associated fibroblasts. (2) T cells. (3) Cytokines. (4) Dendritic cells. (5) DOX enhances T-cell-mediated immunity. (6) DOX targets cancer cell mitochondria. (7) Disruption of mitochondrial function. (8) DOX induces reactive oxygen species (ROS) production. (9) DOX inhibits topoisomerase II, preventing DNA religation. (10) DOX causes DNA damage in cancer cells. (11) Intracellular effects of DOX within cancer cells. (12) DOX action on tumor cells. (13) Cancer-associated macrophages. (14) Cancer cell.

oration method[87]. The nanoparticles are camouflaged by mesenchymal stem cell membrane to deliver both DOX and survivin-targeting siRNA. Success has been demonstrated with DOX/siSUR-PLGA@MSCM NPs, which have improved therapeutic effects in osteosarcoma patients through the combination of a chemotherapeutic drug and gene therapy, owing to their good tumor targeting and biosafety[87]. There are many drugs that also target the tumor microenvironment by altering DNA. Ifosfamide and cisplatin are alkylating agents, and they work in a cell cycle-independent manner in which alkylated DNA adducts lead to DNA damage and cell death[88]. Methotrexate and gemcitabine are examples of antimetabolites, which are mainly S-phase-specific and hinder DNA replication directly or by interfering with deoxyribonucleotide triphosphate synthesis[89]. Etoposide is a type of topoisomerase inhibitor that blocks DNA topoisomerase II (Top2), leading to DNA strand breaks and subsequently perturbations in transcription, replication, and mitosis[90]. A combination of high-dose methotrexate with leucovorin rescue (HDMTX), doxorubicin (Adriamycin), and cisplatin (platin), known as MAP, is the backbone of both neoadjuvant and adjuvant chemotherapy at centers in the United States and most of Europe[91]. These three agents, along with ifosfamide, exhibit single-agent efficacy as well, though they can have a higher effect when used in combination[92]. Also, carboplatin, etoposide, and cyclophosphamide therapies have shown some success in improving outcomes for patients with relapsing disease[93]. Trabectedin is a DNA-binding agent that causes DNA damage and apoptosis and is approved for the treatment of soft tissue sarcoma[94]. Microtubule destabilizing agents such as vincristine are effective in the treatment of solid and hematological tumors, even though not proven in osteosarcoma[95]. Docetaxel in combination with gemcitabine is used

as a second-line treatment for some patients with recurrent osteosarcoma, though with contradictory results, with objective responses reported to range from 0% to 46%[96]. Eribulin is a novel microtubule inhibitor that has been approved for the treatment of some malignancies[97]. The current osteosarcoma treatment consists of preoperative chemotherapy, surgery, and postoperative chemotherapy using high-dose methotrexate, doxorubicin, cisplatin (MAP), ifosfamide, etoposide, cyclophosphamide, and carboplatin[98]. The combination of drugs with different mechanisms of action increases the overall therapeutic efficacy but causes a high rate of complications such as renal and liver damage and bone marrow suppression[99]. It is believed that using a combination of novel anticancer agents with current therapies would result in (i) additive synergistic effects, (ii) reduced toxicity due to dose reduction and (iii) application to metastatic cases with particularly low response rates[100]. As already indicated, chemotherapeutic drugs boost the infiltration of CD8+ T cells and natural killer (NK) cells into tumors, as well as the maturation of antigen-presenting cells (APCs), such as tumor macrophages or dendritic cells[31]. Primary cytostatic and cytotoxic medicines function in this way to restore an immune-reactive tumor microenvironment, which ultimately increases the tumor's susceptibility to immunotherapy[8].

DISCUSSION

The Roles of Immune and Non-Immune TME Components in Doxorubicin Resistance

Immune cells infiltrate into the tumor microenvironment of osteosarcoma. They use different processes, cytokines, and chemicals to attempt to suppress the tumor while providing checkpoints to ensure they do not become dysfunctional or incur harm. The T cells are the major players in tumor cell suppression. They function by eliminating tumor cells through phagocytosis or the direct release of cytokines that destroy cancer cells. Doxorubicin has been shown to sustain these T cells by acting as an immunomodulator, suppressing immunosuppressive cells in the tumor microenvironment. Doxorubicin specifically activates the CD8+ cells, which are cytotoxic to cancer cells while reducing the function of Tregs. The B cells have also been shown to actively participate in tumor suppression. However, these cells are sensitive to doxorubicin. Doxorubicin significantly reduces the amount of B cells present in the tumor microenvironment. B regulatory cells, on the other hand, contribute to doxorubicin resistance through the secretion of cytokines, including IL-10, TGF-β, and IL-35. These cytokines act as a negative regulator of the immune system and contribute to drug resistance. Doxorubicin resistance often implies that there is an altered immune surveillance, where tumor cells continually proliferate instead of dying. Dendritic cells do not directly cause doxorubicin resistance; rather, they promote the influx of Tregs, potential immunosuppressive cells, which inhibit anti-tumor immunity and lead to treatment resistance. After doxorubicin resistance has developed, the resistant tumor cells often produce more immunosuppressive signals, such as myeloid-derived suppressor cells (MDSCs) or TGF-β. This actively induces dysfunction in surrounding dendritic cells, rendering them less effective at presenting antigens to T cells. Tumor-associated macrophages, specifically the M2-polarized, secrete several cytokines that cause immune suppression and doxorubicin resistance. Macrophages generally have the potential to phagocytose doxorubicin in the tumor microenvironment. This makes a minimal amount of doxorubicin available to eliminate tumor cells. There is an interesting process that occurs in the tumor microenvironment mediated by cancer-associated fibroblasts. These cells modify the tumor microenvironment, acting as a physical barrier and secreting cytokines (IL-6, IL-8, CXCL12) that promote stemness and anti-apoptotic pathways. Also, since cancer-associated fibroblasts are responsible for maintaining the extracellular matrix, they increase matrix stiffness by depositing abnormal levels of collagen types I and IV and hyaluronic acid, creating a physical barrier that restricts drug penetration into the tumor. In this case, doxorubicin is unable to penetrate into the tumor microenvironment to restrict tumor cells.

How Does DOX-Induced Immunomodulation Promote Both Tumor Control and Immune Escape?

Generally, changes in the tumor microenvironment (TME) are marked by hypoxia, acidity (low pH), high interstitial fluid pressure and the presence of immunosuppressive cells. The significance is that there is a decrease in doxorubicin efficacy and delivery, and an increase in its toxicity. The tumor microenvironment often acts as a physical and biological barrier that induces resistance, but it can also be leveraged to trigger targeted drug release and subsequent tumor suppression. It should be understood that the metabolism of doxorubicin in the body, coupled with specific changes in the tumor microenvironment, can either promote

or suppress the tumor. After doxorubicin resistance, the resistant cells tend to induce immunosuppression, enhance angiogenesis, alter metabolism and pH, and increase fibrosis and stemness. The resistant cells also secrete factors that activate myeloid-derived suppressor cells (MDSCs). In this state, an immunosuppressive environment is once again created, protecting the tumor cells. Enhanced angiogenesis is driven by sustained secretion of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). The aggressive growth of tumor cells induces hypoxia, and the tumor microenvironment becomes more acidic due to the conversion of pyruvate to lactic acid and subsequent ATPs. This, in turn, promotes drug efflux and hinders the efficacy of any doxorubicin therapy. Drug efflux is also driven by tumor cell stemness.

Comparing Osteosarcoma Findings with Data from Other Solid Tumors

Osteosarcoma has always been a subject of interest to researchers. Compared to other solid tumors like colorectal cancer, osteosarcoma cannot be linked to a specific mutation or cause. Studies in the literature only associate the cause with random genetic mutations and environmental factors. Another unique feature observed in the osteosarcoma microenvironment is the infiltration of immune cells. The immune modulation in the osteosarcoma tumor microenvironment is most commonly referred to as “cold” or “immune-excluded”, as the microenvironment is dominated by tumor-associated macrophages (TAMs), which promote a highly immunosuppressive niche that facilitates immune evasion. Other solid tumors, like melanoma and lung cancer, are heavily infiltrated with T cells. In view of this, resistance to chemotherapy and recurrence after a primary event are predicted to be intrinsically possible. As reported in the National Institutes of Health (NIH)[6], a significant portion of osteosarcoma patients (often over 30%) do not respond well to initial neoadjuvant chemotherapy, indicating a high level of innate resistance that is less common in some other pediatric solid tumors. There is also a high chance of multiple drug resistance. Extracellular vesicles, known as exosomes, are used by many tumors to signal, but in osteosarcoma, exosomes are specifically used to actively transport chemotherapeutic drugs out of tumor cells, contributing significantly to multiple drug resistance. Varieties of structurally unrelated chemotherapeutic drugs with different mechanism of actions are shown to be inclusive. P-glycoprotein (P-gp), a transporter, is highly expressed in the osteosarcoma tumor microenvironment and also contributes to drug efflux. Both osteosarcoma and colorectal cancer are very stiff; colorectal cancer is known for its dense fibrotic barrier, while osteosarcoma has an osteoid barrier largely due to the production of osteoid matrix, abnormal collagen, and fibronection. The dense matrix of the tumor microenvironment protects tumor cells from doxorubicin therapy. Again, in common with colorectal cancer, osteosarcoma exhibits significant stemness, which is highly efficient in DNA repair and detoxification, allowing it to survive high-dose chemotherapy.

Current Limitations on Osteosarcoma Research

A well-known limitation of studies on osteosarcoma is the challenge of effectively targeting the tumor microenvironment without side effects. In the tumor microenvironment, there exist the anti-tumor suppressant immune cells, non-immune cells, and cytokines, which are also subjected to the cytotoxic attack of doxorubicin. At high doses of doxorubicin, these components decline, and at low doses, tumor cells survive. Another setback has been the difficulty in conducting research on both primary and secondary osteosarcoma in the same patient. The occurrence of the primary cancer with no further studies on the recurrence in the same patient has been a challenge. Therefore, there is a lack of longitudinal monitoring. This can be understood because it is somewhat not possible to predict if a patient with a primary tumor could possibly experience a recurrence. Some patients with the primary cancer do not experience recurrence later in life despite all odds. This has made it difficult to compare primary and secondary osteosarcoma in the same patient. The exact pattern the cancer takes is highly unpredictable as a result. Predicting when and how resistance to doxorubicin therapy and other chemotherapies becomes incurable has also been a challenge for researchers. Resistance has been documented to be highly likely, but the timing of when no chemotherapeutic agent, even at high doses, becomes ineffective against tumor cells has not been studied. The concept of the tumor microenvironment highlights the roles of its components and their impact on immunotherapy. In knowing the role each component plays, researchers focus on autoimmunity, where the body’s own immune system is used as a driving force to suppress tumor cells while causing fewer effects. However, the proportions of each component must be considered, as this could be the main pivot for cancer cell suppression and elimination. Considering the frequency of relapse and drug resistance, the essence of immunotherapy becomes paramount.

CONCLUSIONS

Doxorubicin therapy initially interacts with elements of the tumor microenvironment, whereas the subsequent changes it induces subsequently impact these elements. By secreting interleukins and tumor growth factors, Tregs and Bregs establish an immunosuppressive niche in the tumor microenvironment, which exacerbates doxorubicin resistance. Tumor cells develop resistance to apoptosis caused by doxorubicin. Large doses of doxorubicin can be phagocytosed by macrophages, reducing the quantity available to kill tumor cells. Dendritic cells that are resistant to doxorubicin lose their ability to deliver antigens to T cells, which is crucial. CAFs create more aberrant collagen, which thickens the tumor microenvironment and acts as a barrier to stop doxorubicin from penetrating. Due to its cardiotoxicity, doxorubicin must be administered in small doses, leaving some cancer cells alive. This means that although doxorubicin alters the tumor microenvironment, some osteosarcoma cells can withstand chemotherapy and then proliferate, leading to recurrence, either shortly after treatment or later in patients' lives. There is a need for a system that provides comprehensive care for a patient throughout their life, as sole reliance on chemotherapy can lead to serious side effects, including cardiotoxicity. Since the patient's immune cells are employed to inhibit and eradicate the cancer, immunotherapy is a potential treatment.

Authors' Contributions:

Conception: AAAQ, IU, AA and OEI. Writing-original draft preparation: AAAQ, OEI, and IU. Writing-review and editing: AAAQ, IU, AA and OEI. Validation: AAAQ, IU, and OEI. Supervision: IU, SS, and IR. All authors read and approved the final manuscript.

Conflict of Interest:

The authors declare no conflict of interest.

Funding:

The research was carried out within the framework of the state assignment of the Ministry of Health of the Russian Federation (No. NZAF-2024-0025).

Ethics Approval and Informed Consent:

Not applicable due to the design of the study.

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